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13. ABSTRACT *(Maximum 200 Words)*

Uncontrolled cell proliferation is a predominant feature of cancer. In fact, it is the deregulation of signaling pathways that control cell proliferation that lead to the course of oncogenic transformation and tumorigenesis. The ErbB families of growth factor receptors (ErbB1-4) and their ligands have been strongly implicated in the genesis of a number of human carcinomas. The ErbB receptors mediate cellular responses to growth factors through their intracellar signaling domain. Therefore, it is critical to identify and characterize the effectors associated with the phosphorylated C-terminal tail of activated ErbBs and the effectors mediated the ErbB-dependent signals to the downstream mitogenic signaling pathways such as BMK1 cascade. For this one-year project, we have systematically fished out effectors that interact with the signaling domains of ErbBs and molecules that bind to MEKK3, the upstream regulating kinase for BMK1 pathway by yeast two-hybrid system. In addition, we have generating recombinant proteins of some of these interacting protein and establishing binding assays for analyzing their binding to the targeted molecules in mammalian cells. These studies should provide fundamental information about the mechanism of ErbB-mediated breast cancer development and could offer new therapeutic strategies for preventing the progression of breast cancer in the clinic.

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Table of Contents

Introduction:

Growth factor receptors belonging to the Erb family (ErbB1-4) are crucial mediators of uncontrolled growth and metastasis of certain mammary tumors. The most notable family member is the receptor ErbB2 which is overexpressed in approximately 30% of human breast cancer cases. Patients with tumors that overexpress ErbB2 have a shorter relapse time and a lower overall survival rate compared to patients whose tumors do not overexpress ErbB2. In fact, transgenic mice that overexpress either wild type or oncogenic forms of the ErbB2 gene in the mammary epithelia develop mammary tumors that frequently metastasize to other organs.

The physiologic ligand for ErbB2 is not known, but this receptor has been shown to act as the preferred heterodimeric partner for other ErbBs. Among the heterodimers formed by ErbB2, the ErbB2-ErbB3 heterodimer appears to generate the most potent mitogenic and oncogenic signals. It is the C-terminal phosphorylation domain of ErbB3 that is responsible for generating these especially potent mitogenic signals. Since ErbB3 elicits hyper-activated signaling through the ErbB-signaling network, deregulation of its function likely results in diverse neoplastic events. In fact, several lines of evidence implicate ErbB3 as a key player in both the oncogenesis and chemoresistance of human cancers: (1) Coexpression of ErbB3 with ErbB2 substantially enhances the resistance of breast cancer cells to various anti-cancer drugs. (2) Increased expression and activity of ErbB3 has been demonstrated in certain human mammary tumor cell lines and in human breast cancer. (3) Overexpression of ErbB3 has also been observed in epidermoid carcinoma of the larynx as well as esophageal carcinoma. (4) In oral squamous cell carcinoma, high expression of ErbB3 correlates with the presence of lymph node metastasis, survival rate and mode of invasion. Thus, the ErbB3 signaling pathway provides a potential drug target for intervening in the progression of certain human cancers.

Recent advances have demonstrated that ErbB3 is closely involved in proliferation and metastasis of certain human cancers. It is through the phosphorylated cytoplasmic domain that ErbB3 recruits a repertoire of signaling proteins that subsequently pass down proliferative and sometimes oncogenic signals. To date, signaling molecules associated with activated ErbB3 remain incompletely identified and characterized. In this regard, our lab has focused our effort on improving our knowledge of the cellular mechanisms regulating or mediating ErbB3 activity. To this end, we devised a screening method for isolating intracellular effectors that physically interact with activated ErbB3. Using this approach, we have discovered several novel cellular proteins that bind to the signaling domain of activated ErbB3. We hypothesize that these effectors mediate and/or regulate the cancerous activity of ErbB3. Herein, we propose to study the role and the molecular mechanism of action of these effectors of ErbB3-signaling. In addition to this, we have also isolated intracellular molecules that interact with MEKK3 which is one of the crucial element in ErbB-dependent mitogenic signaling. These studies will provide fundamental knowledge of ErbB-dependent mammary tumorigenesis and which may be useful in the design of new strategies for preventing the development of breast cancer.

Body:

Design of activated ErbB3 signaling tails to act as bait in yeast-two hybrid screens. To identify proteins that interact with phosphorylated docking sites of ErbB3, investigators have previously tested the binding of known proteins, that contain phospho-tyrosine binding (PTB) or SH2 domains, to activated ErbB3 using *in vivo* or *in vitro* binding assays [1-3]. This approach has identified some ErbB3 associated proteins. In an effort to systematically uncover novel effectors that interact with activated ErbB3, we designed a library screening approach based on the yeast two-hybrid system. To this end, we engineered yeast two-hybrid baits containing ErbB3 signaling tail with varing levels of tyrosine phosphoryation (Figure 1). ErbB3 requires phosphorylation from another ErbB family member in order to signal as it lacks its own tyrosine kinase activity due to the substitution of critical amino acid residues within its otherwise conserved tyrosine kinase domain [4]. Given the substantial similarity within the tyrosine kinase (TK) region among the ErbBs family, we are able to substitute the kinase domain of ErbB3 with the corresponding functional TK domain from ErbB1, 2 or 4. These chimeras were then fused in frame with the GAL4 DNA binding domain of the yeast expression vector pGBKT7 (Clontech) to be used as baits in subsequent screenings (Figure 1). We analyzed the level of tyrosine phosphorylation in yeast for each kind of bait we constructed using the anti-phosphotyrosine antibody PY20 (Santa Cruz). As shown in Figure 2, we found that the ErbB4/3 bait had the highest level of tyrosine phosphorylation, ErbB2/3 was moderately phosphorylated, and that phosphorylation of ErbB1/3 as well as ErbB3 (wt) were both undetectable.

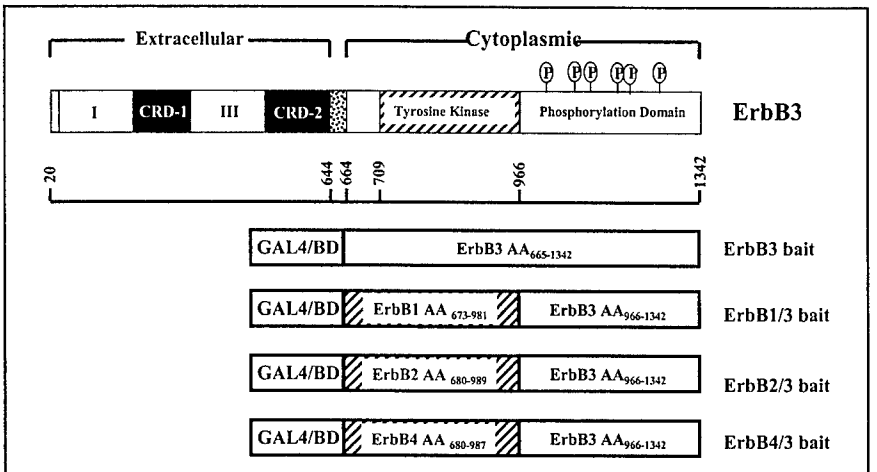

Figure 1: Yeast two-hybrid bait constructs expressing the C-terminal tail of ErbB3. Yeast expression plasmids were constructed to encode fusion proteins between the GAL4 binding domain (GAL4/AD) and the cytoplasmic domain of ErbB3 as indicated. In constructs ErbB1/3, ErbB2/3 or ErbB4/3, the kinase domain of ErbB3 was replaced with the kinase domain of ErbB1, ErbB2 or ErbB4 as shown. These constructs are not drawn to the proportion of their actual sizes.

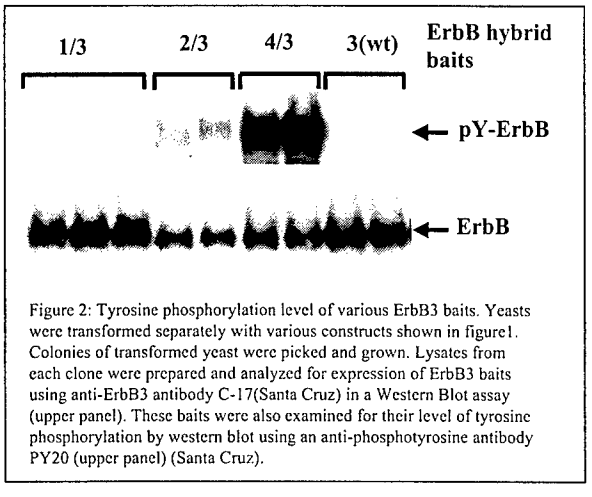

Figure 2: Tyrosine phosphorylation level of various ErbB3 baits. Yeasts were transformed separately with various constructs shown in figure1. Colonies of transformed yeast were picked and grown. Lysates from each clone were prepared and analyzed for expression of ErbB3 baits using anti-ErbB3 antibody C-17(Santa Cruz) in a Western Blot assay (upper panel). These baits were also examined for their level of tyrosine phosphorylation by western blot using an anti-phosphotyrosine antibody PY20 (upper panel) (Santa Cruz).

Identification of intracellular proteins associated with tyrosine-phosphorylated ErbB3 baits. Both ErbB2/3 and ErbB4/3 chimeras were independently used as baits in the following library screenings. A total of 5.3 X 10^7 transformants were screened from a human epithelial carcinoma cDNA library and 152 positive clones were selected for sequence analysis. These positive cDNA clones were chosen based on their potential interaction with bait which gave rise to transformed yeast that grew in media lacking histidine and adenine. To verify whether the phosphorylation of our bait is required for the binding of these potential proteins we tested the affinity of each clone for the various ErbB3 baits. A number of proteins only interact with phosphorylated ErbB4/3 or ErbB2/3 baits but not with ErbB3 (wt) bait which is not tyrosine phosphorylated. One such clone, CL68, is shown in figure 3. The BLAST algorithm and the nucleotide database at the National Library of Medicine revealed that a number of these positive clones contain SH2 domains. We have classified these clonses into 3 groups (Table 1). Group 1: known ErbB3 binding proteins whose functions have already been described. Group 2: proteins that were described previously but whose role in ErbB3-signaling is not known. Group 3: novel proteins whose sequence and function has never been described. Among the molecules we isolated, we are interested in studying VAV3.1, CL7 and CL68 whose primary structures are shown in figure 4. Full-length cDNA of VAV3.1 and CL68 were acquired by screening a human carcinoma cDNA library, and we are currently in the process of isolating a full-length cDNA clone of CL7. VAV3.1 is a major splicing variant of VAV3 [5], which belongs to the VAV oncogene family and has strong homology to the other family members VAV and VAV2 [6;7]. VAV3 protein has eight functional domains including a Dbl homology domain, a Pleckstrin homology, a calponin homology region, an acidic motif, a zinc finger region and a C-terminal SH3-SH2-SH3 region [6;7]. VAV3 has been

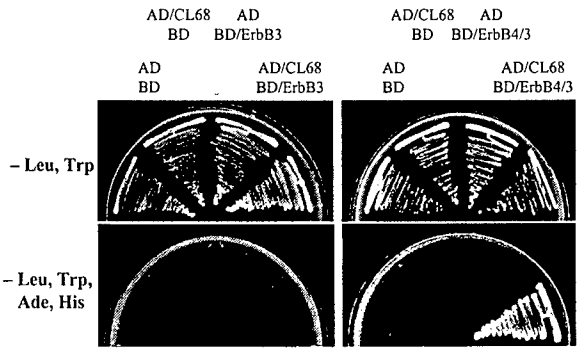

Figure 3: CL68 physically interacts with tyrosine-phosphorylated ErbB4/3 bait but not with unphosphorylated ErbB3 bait in the yeast two-hybrid system. The yeast strain PJ69-2A was cotransformed with vectors encoding the GAL4 DNA binding domain (BD) fusion protein (BD/cytoplasmic domain of ErbB3 or ErbB4/3) along with the indicated GAL4 activating domain (AD) fusion protein CL68. Transformed yeast was cultured on plates containing the indicated selective media.

Group #1	Grb7 (containing SH2 motif)
	p85 α (PI-3K subunit, containing SH2 and SH3)
	p85 β (PI-3K subunit, containing SH2 and SH3)
Group #2	p55 γ (PI-3K subunit, containing SH2 motif)
	VAV3.1 (containing SH2 and SH3 motifs)
Group #3	CL7 (containing SH2 motif)
	CL68 (containing SH2 motif)

Table 1: SH2-motif containing proteins isolated from yeast two-hybrid screens using ErbB2/3 or ErbB4/3 baits.

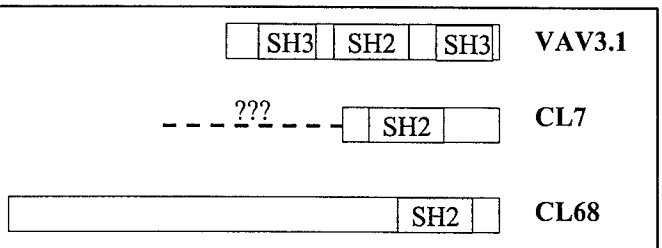

Figure 4: Primary structures and functional motifs of VAV3.1, CL7 and CL68.

implicated in signaling and cytoskeletal pathways through binding and subsequent activating of Rho GTP-binding proteins. Conversely, the biological function of VAV3.1 is completely unknown as it only possesses the C-terminal SH3-SH2-SH3 domain of VAV3 [5]. Thus, VAV3.1 may share some similar functions with VAV3 or it may act by competing with normal VAV3 function. To date, the sequences and biological function of both CL7 and CL68 have not been reported. Since they both contain an SH2 motif, we believe that they bind to activated ErbB3 by their SH2 motifs, and participate in subsequent signaling events. CL68 protein consists of ~450 amino acid residues with one conserved SH2 motif in its C-terminal end. The remainder of the CL68 protein shows no similarity to any other known proteins and has no identifiable functional motifs. Thus, the role of CL68 in ErbB3 signaling remains to be characterized. The sequence of the partial cDNA clone CL7 reveals no similarity with other known proteins other than its C-terminal SH2 domain. We will obtain more information about CL7 after acquiring its complete cDNA sequence.

Binding of VAV3.1, CL68 and CL7 with activated ErbB3 in mammalian cells. The intracellular signaling region of ErbB3 is trans-phosphorylated by its heterodimeric partner upon engagement with the corresponding ligand. To verify that the proteins

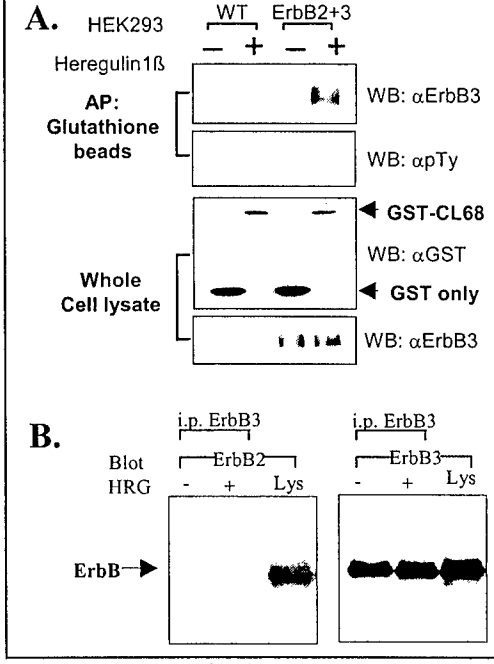

Figure 5: CL68 protein copurified with phosphorylated ErbB3 in mammalian cells. **A.** HEK293 cells or HEK293(ErbB2+3) were transfected with a vector encoding GST-tagged CL68 or GST alone as indicated. After transfection, cell lysates were prepared, affinity purified by Glutathione beads, and analyzed for copurification with ErbB3 by western blotting using anti-ErbB3 antibody C-17 (San Cruz) (first panel). Same samples were tested for tyrosine phosphorylation using anti phosphotyrosine antibody PY20 (Transduction Lab) (second panel). Western blot analysis of whole cell lysates using antibodies against GST tag (third panel) and ErbB3 (fourth panel) confirmed the expression of ErbB3 and GST tagged proteins in transfected HEK293 cells. **B.** ErbB receptors in heregulin-treated HEK293(2+3) cells are not co-immunoprecipitated by heterodimer formation. Under the same lysis conditions as used in panel A, ErbB3 receptor was immunoprecipitated from HEK293(2+3) and the resulting complexes were Western blotted with the indicated antibodies. The whole cell lysates in the very right lanes of each blot served as a positive control for ErbB2 or ErbB3 expression.

encoded by the isolated clones actually associate with such an activated ErbB3, GST-tagged CL68 was transiently expressed in HEK293 cells stably expressing ErbB2 and ErbB3. After transfection, these HEK293(ErbB2+3) cells were treated with heregulin for 10 mins, and cell lysates were prepared followed by affinity purification using

Glutathione-Sepharose beads. We found GST-tagged CL68 protein co-purified with tyrosine-phosphorylated ErbB3 (Figure 4A). To establish whether CL68 interacts with ErbB3 or with heterodimers between ErbB3 and ErbB2, the stability of the ErbB2-ErbB3 heterodimer complex was investigated under our lysis conditions (Figure 4B). ErbB2 was not detected in Western blots of ErbB3 immunocomplexes from heregulin-stimulated HEK293(ErbB2+3) cells. These results support the findings from the yeast two-hybrid screen and indicate that the CL68 SH2 domain mediates its association with phosphorylated ErbB3. However, unlike the yeast two hybrid results, this experiment in mammalian cells cannot exclude the involvement of other cellular proteins mediating the association of CL68 with ErbB3. In other words, it remains possible that the co-purification of CL68 with phosphorylated ErbB3 is not direct and may be mediated through other cellular proteins. To clarify this issue we propose, in section D1, to carry out Far-Western Assays to test the direct binding of these SH2-containing molecules with phosphorylated ErbB3.

Key Research Accomplishments:

1. Identifying signaling molecules interacting with activated ErbB.
2. Identifying effectors mediating ErbB-dependent intracellular signaling.

Reportable outcomes:

Due to support of this one-year fund, we have gathered experimental data for applying funding of National Cancer Institute, and the proposal was consequently funded.

Conclusions:

We have identified novel effectors that may play a role in mediated ErbB-dependent oncogenic signaling. Since ErbBs is involved in mammary carcinogenesis, our finding may aid in the development of novel strategies that inhibit the uncontrolled growth of human breast cancer cells.

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Appendices:

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